METHOD VALIDATION FOR THE QUANTIFICATION OF PFOS AND PFOA IN FISH

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Introduction

Perfluorinated compounds (PFCs) exhibit favourable chemical characteristics that has led to their wide spread use (industrial applications) as well as their ubiquitous presence in the environment. Contrary to the bio-accumulative pattern of most persistent organic pollutants (POPs) that partition into fatty tissues, PFCs bind to proteins[1]. This property is of specific concern in protein rich food such as meat and fish a major constituent of the South African diet. Currently there is only limited data available on the presence of these compounds within the South African environment, in part, due to the lack of analytical capacity. Therefore, an analytical method was developed using high pressure liquid chromatography tandem mass spectrometry (HPLC-MS/MS) for the analysis of perflurooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in fish tissue. The following study details the key method validation criteria that could influence the quantification results. Validation criteria that were assessed included selectivity/specificity, accuracy, precision, linearity, working range, limit of detection (LOD), limit of quantification (LOQ), ruggedness and robustness.

Materials and methods

The analytical method was developed using authentic standards obtained from Cambridge Isotope Laboratories. Although not quantified, 10 additional PFCs were included in the analysis for screening purposes, namely perfluorobutanesulfonic acid (PFBS), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTrDA) and perfluototetradecanoic acid (PFTA). Prior to use, all equipment and consumables were pre-cleaned with methanol (MeOH). To decrease the risk of contamination, high density polypropylene (HDPP) consumables were used. Additionally, various blanks were run throughout the analytical procedure to evaluate PFC contamination not only from the extraction and clean-up method but also from the LC-MS/MS system.

A 1g subsample of homogenised fish fillet was gravimetrically spiked with C13 isotopically labelled internal standard, M8PFOA, and M8PFOS. The spiked samples were then allowed to equilibrate overnight. Quality control samples were spiked with native and labelled standards and allowed to equilibrate overnight. These samples were then extracted in duplicate with MeOH. The pooled supernatant was dried at 40°C under a gentle stream of nitrogen prior to dispersive solid phase extraction with activated carbon (EnviroCarb^{TM}). The extracts were filtered (0.22 µm nylon filter), evaporated and reconstituted in 250 µL of 40:60 MeOH: aqueous 5 mM ammonium formate prior to analysis. The chromatographic system used was an Agilent 1100 Series HPLC coupled to a Waters Micromass Quattro Micro electrospray ionization tandem MS. Analytes were separated on a fluorinated reverse phase column, FluoroSep RP-Octyl (5 µm x 150 mm x 2.0 mm i.d.). The instrumental conditions were adapted and optimised from literature[2,3]. The quantification approach employed was isotope dilution mass spectrometry (IDMS). The LOD and LOQ were determined by linear regression where S_a was defined as the intercept and the LOD was defined as three times the standard deviation of $S_{y/x}$ and the LOQ was defined as ten times the standard deviation of $S_{y/x}$. Recovery was assessed by gravimetrically spiking blank samples. An eight point calibration curve was constructed for quantification using a matrix-matched calibration curve. Matrix-matched calibration curves were constructed using extracted blank matrix samples spiked with a known amount of native and labelled PFOS and PFOA. For a calibration curve to be used for quantification a R^2 greater than 0.99 had to be obtained. A blank matrix sample, method blank and spiked matrix matched recovery control samples were included in each batch to ensure the quality of the extraction and analytical runs.

Results and discussion

Method validation is a scientific process in which analytical data obtained is critically evaluated to ensure that the developed method is fit for its intended purpose and meets client/ research objectives. This therefore ensures that environmental data generated in different laboratories remains relatively comparable. For the purpose of this

paper selectivity/ specificity, working range, LOD/LOQ, accuracy, bias, precision, recovery and uncertainty will be discussed.

Selectivity/Specificity: Native and isotopically labelled standards were infused into the MS to obtain multiple reaction monitoring (MRM) transitions for each compound while optimizing the cone voltage and collision energy. Thereafter individual standards were run to ensure optimal separation. Ten PFCs, PFTA, PFTrDA, PFNA, PFDA, PFUnA, PFDoA, PFHpA, PFOA, PFOS, PFBS, PFHxA and PFHxS, commonly found in environmental samples were chromatographically separated (**Figure 1**). During method development, the interference sodium taurodeoxycholate hydrate (TDCA), a cholic acid known to co-elute with PFOS, was separated from PFOS to prevent possible bias. Additionally, the experimental isotope profile obtained for PFOS was verified against the theoretical model to ensure the compound was correctly identified.

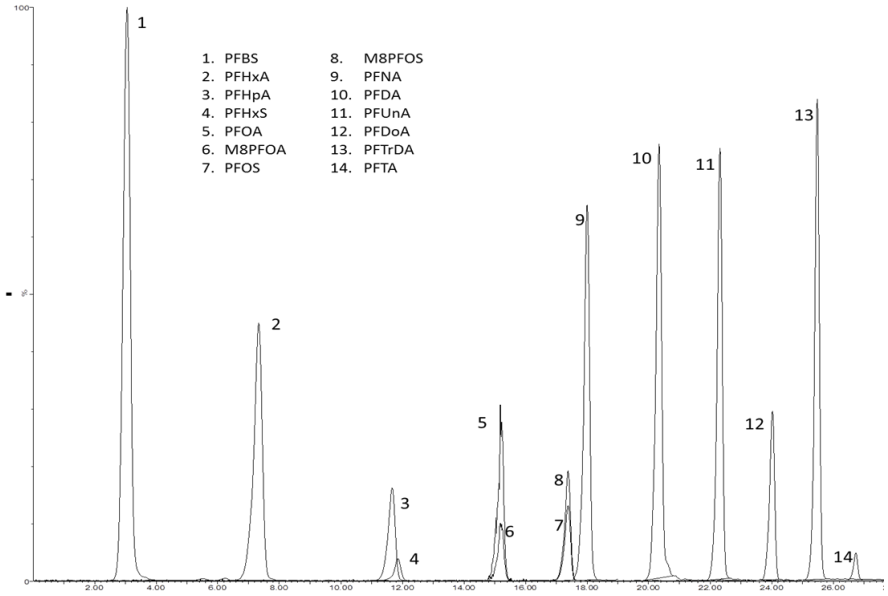

Figure 1: Chromatographic separation of the selected PFCs screened during the analysis, as well as the quantified PFOA and PFOS together with their isotopically labelled standards M8PFOA and M8PFOS

Working linear range/LOD/LOQ/Sensitivity/Linearity: The LOD and LOQ were determined by linear regression as discussed in materials and methods (**Figure 2**). The LOD for PFOA and PFOS in fish was calculated as 17 and 8 ng/g, and the LOQ as 56 and 26 ng/g, respectively.

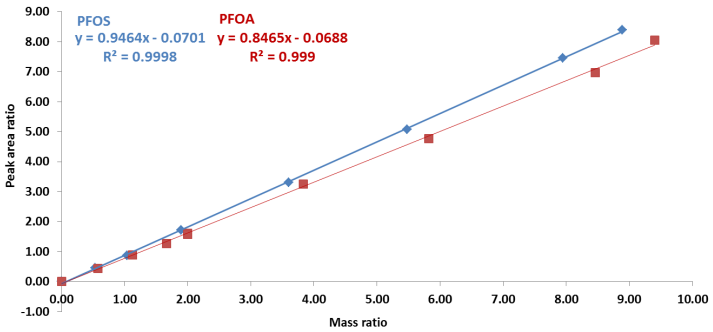

Figure 2: Matrix matched calibration curve generated for PFOA and PFOS (peak ratio – peak area native/peak area labelled; mass ratio – mass of native/mass of labelled)

Accuracy, bias and precision: The measurement accuracy and precision was assessed by analysing gravimetrically spiked fish matrix that had been previously screened for the presence of PFCs. These repeat analyses were performed over a two week period and also provided an initial indication of the method reproducibility. For both analytes repeat measurements fell within the calculated measurement uncertainty as illustrated in **Figures 3a** and **b**.

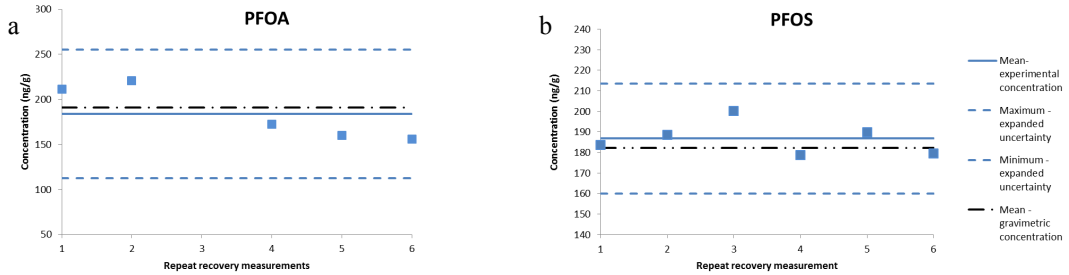

Figure 3: Repeat measurement results (ng/g) obtained for samples spiked with PFOA (a) and PFOS (b) compared to the gravimetric concentration

Recovery: The recovery was determined, using matrix matched spiked samples, for all twelve PFCs analysed. Initial recovery test indicated an absolute recovery of the native compound ranging from 83 – 150%. The absolute recovery for PFOA and PFOS throughout all analysis performed varied between 50 – 140%. Although the use of isotopically labelled internal standards can assist in compensating for many of the variations in recovery, including losses during sample extraction and clean-up, variations in the injection volumes and sample dependent matrix enhancement or suppression during electrospray ionisation; it does not account for recovery efficiency. Recovery efficiency can vary due to sample dependant factors or due to the fact that the labelled and native standards are not fully equilibrated with the material. Therefore recovery bias and variability was the greatest contributor to the overall measurement uncertainty.

Uncertainty: The factors affecting uncertainty in persistent organic pollutant (POP) measurements can be summarized in the Ishikawa diagram (**Figure 4**). From this diagram the main contributors to measurement uncertainty can be summarized in the following factors, namely calibrant purity, uncertainty associated with assigned standard concentration, measurement precision determined as the %RSD, recovery bias and error of the calibration curve.

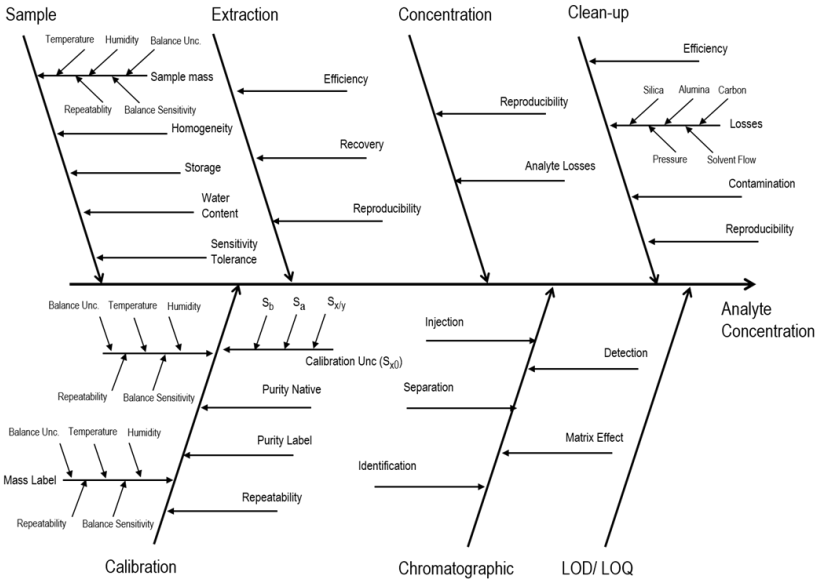

Figure 4: Ishikawa diagram illustrating the uncertainty contributors for the analysis of POPs in abiotic and biotic matrices

The uncertainty calculations for PFOA and PFOS are summarised in **Tables 1 and 2**. Six repeat spiked samples were used to calculate the precision uncertainty as well as the bias associated with recovery. The relative uncertainty calculated for PFOA was 38% with the largest uncertainty contribution originating from recovery variation. The relative uncertainty for PFOS was calculated as 15% with the largest contribution imported from the purity of the native standard used during quantification.

Table 1: Uncertainty calculations for the quantification of PFOA

PFOA		**x**	**u**	**u/x**	**u/x^2**
Purity	Purity of the standard used for quantification	0.9800	0.0115	0.0118	0.0001
Concentration of standard	Assigned value	47.8000	2.4000	0.0502	0.0025
Precision	%RSD of the 6 repeat measurements	184.1087	16.2642	0.0883	0.0078
Bias	Variation in recovery	184.1087	29.6236	0.1609	0.0259
S_{xO}	Error of the calibration curve	184.1087	6.5708	0.0357	0.0013

Minimum ng/g	112.68	Uncertainty	0.04	
Maximum ng/g	255.53	U – Combined standard uncertainty	35.71	ng/g
LOD (ng/g)	16.78	U (k = 2) – Expanded uncertainty	71.43	ng/g
LOQ (ng/g)	55.92	Rel U – Relative uncertainty	**38.80**	%

Table 2: Uncertainty calculations for the quantification of PFOs

PFOS		**x**	**u**	**u/x**	**u/x^2**
Purity	Purity of the standard used for quantification	0.9800	0.0115	0.0118	0.0001
Concentration of standard	Assigned value	47.8000	2.4000	0.0502	0.0025
Precision	%RSD of the 6 repeat measurements	186.7500	4.2837	0.0229	0.0005
Bias	Variation in recovery	186.7500	7.6362	0.0409	0.0017
SxO	Error of the calibration curve	186.7500	2.9883	0.0160	0.0003

Minimum ng/g	160.04	Uncertainty	0.01	
Maximum ng/g	213.46	U – Combined standard uncertainty	13.35	ng/g
LOD (ng/g)	7.92	U (k = 2) – Expanded uncertainty	26.71	ng/g
LOQ (ng/g)	26.39	Rel U – Relative uncertainty	**14.30**	%

Acknowledgements
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